Published in final edited form as:
Hypertension. 2011 August ; 58(2): 303–309. doi:10.1161/HYPERTENSIONAHA.110.166819.

Exacerbated pulmonary arterial hypertension and right ventricular hypertrophy in animals with loss of function of extracellular superoxide dismutase

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Abstract

Studies have demonstrated that increased oxidative stress contributes to the pathogenesis and the development of pulmonary artery hypertension (PAH). Extracellular superoxide dismutase (SOD3) is essential for removing extracellular superoxide anions and it is highly expressed in lung tissue. However, it is not clear whether endogenous SOD3 can influence the development of PAH. Here we examined the effect of SOD3 knockout on hypoxia-induced PAH in mice and a loss-of-function SOD3 gene mutation ($SOD3^{E124D}$) on monocrotaline (40 mg/kg)-induced PAH in rats. SOD3 knockout significantly exacerbated 2 weeks hypoxia-induced right ventricular (RV) pressure and RV hypertrophy, while RV pressure in SOD3 KO mice under normoxic conditions is similar to wild type controls. In untreated control rats at age of 8 weeks, there was no significant

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Disclosures

Conflict of Interest Disclosures: Dr. Eric Ostertag has served as the Chief Executive Officer of Transposagen Biopharmaceuticals; Dr. Aron Geurts has served as a consultant for Transposagen Biopharmaceuticals. The other authors report no conflicts.

difference between wild type and SOD3^{E124D} rats in RV pressure and the ratio of RV weight to left ventricular weight (0.25±0.02 in wild type rats vs. 0.25±0.01 in SOD3^{E124D} rats). However, monocrotaline caused significantly greater increases of RV pressure in SOD3^{E124D} rats (48.6±1.8 mmHg in wild type vs. 57.5±3.1 mmHg in SOD3^{E124D} rats), of the ratio of RV weight to left ventricular weight (0.41±0.01 vs. 0.50±0.09, $p<0.05$), and of the percentage of fully muscularized small arterioles in SOD3^{E124D} rats (55.2±2.3 % vs. 69.9 ±2.6 %, $p<0.05$). Together, these findings indicate that the endogenous SOD3 has no role in the development of PAH under control conditions, but plays an important role in protecting the lung from the development of PAH under stress conditions.

Keywords

pulmonary artery hypertension; right ventricular hypertrophy; oxidative stress; extracellular SOD

Introduction

Pulmonary artery hypertension (PAH) is a progressive disease with a very poor prognosis. PAH is characterized by a progressive elevation of pulmonary arterial pressure, ultimately inducing right ventricular (RV) hypertrophy and heart failure. Studies have demonstrated that increased oxidative stress, such as enhanced production of superoxide anions and other reactive oxygen species may contribute to the pathogenesis and the development of idiopathic PAH (IPAH) in patients and to PAH secondary to high pulmonary vascular flow in lambs.[1,2] In addition, administration of antioxidants attenuates the development of PAH,[3,4] suggesting that pulmonary oxidative stress regulates the development of PAH.

Superoxide dismutase (SOD) is a first line of defense against free radical attack. Three SOD isozymes have been identified, including a copper/zinc-containing SOD (SOD1), which is primarily cytosolic in location, a mitochondrial manganese SOD (SOD2), and an extracellular SOD (SOD3). SOD3 is a glycoprotein secreted into the extracellular fluid by fibroblasts that binds to sulfated polysaccharides, such as heparin and heparan sulfate,[5,6] as well as to other matrix components.[7,8] As a result, SOD3 binds to the surface of endothelial cells and the extracellular matrix, which has a high abundance of heparan sulfate.[9] Lung is one of the organs with a relatively high SOD3 expression.[5,10,11] Previous studies have demonstrated that overexpression of SOD3 attenuates hypoxia-induced PAH in mice[11] and monocrotaline-induced PAH in rats.[12] Overexpression of SOD3 also attenuates bleomycin-induced lung injury.[13] However, because SOD3 has a minimal impact on total tissue SOD activity,[10] it is uncertain whether endogenous SOD3 can influence the development of PAH. To address this question, we examined the effect of SOD3 knockout (KO) on hypoxia-induced PAH in mice, and the effect of SOD3 gene mutation (SOD3^{E124D}) on monocrotaline-induced PAH in rats. Here we report that both SOD3^{E124D} in rats or SOD3 knockout in mice had no effect on PAH and RV hypertrophy under control conditions but resulted in significantly greater increases of right ventricular pressure, pulmonary vascular remodeling, as well as greater RV hypertrophy in response to monocrotaline or chronic hypoxia. The findings indicate that the endogenous SOD3 plays an important role in protecting the lung from the development of PAH and RV hypertrophy under stress conditions.

Materials and Methods

SOD3 knockout Mice

SOD3 knockout mice and control wild type mice used in the present study are described previously.[6,14,15] This study was approved by the Institutional Animal Care and Use Committee of University of Minnesota.

SOD3^{E124D} rats

SOD3^{E124D} (SS-Sod3^{m1Mcwi}) rats were identified as a mutation in an ethylnitrosourea (ENU) mutagenesis screen by the PhysGen Program in Genomic Applications (http://pga.mcw.edu), backcrossed to the *Dahl/Salt Sensitive* (Dahl/SS) strain, intercrossed and maintained as a homozygous colony. N2F7-F8 generation animals were provided as a gift by Transposagen Biopharmaceuticals. Since these rats were intentionally generated in the Dahl/SS background, provided by the Medical College of Wisconsin to Charles River Laboratory, Dahl/SS rats were used as controls.

Hypoxia-induced PAH in mice

Male SOD3 knockout mice and wild type control mice at age 10–14 weeks were exposed to hypobaric hypoxia as described by Hampl et al.[16] Briefly, the pressure in the chamber was decreased progressively from 0.8 atm (16.9% O2) on Day 1 to 0.5 atm (10.5% O2) after Day 7, and was maintained at 10.5% O2 for 2 more weeks. The chamber was opened once every week for cleaning and feeding. After exposure to 10.5% O2 for 2 weeks, mice were removed from the hypoxia chamber for determination of RV pressure and hypertrophy. The sham mice were kept in normobaric conditions.

Induction of PAH in rats with monocrotaline (MCT)

Male SOD3^{E124D} rats and wild type control rats (Dahl/SS) at age 5 weeks were given intraperitoneal injections of MCT (40 mg/kg, Sigma, St. Louis, MO) or an equivalent volume of vehicle as a control. The bodyweight of these rats were monitored weekly. Right ventricular (RV) and aortic pressure were then determined at 3 weeks after MCT or vehicle injection and samples were collected for related tests.

Having noticed that MCT-induced more PAH and pulmonary vascular remodeling in SOD3^{E124D} rats, we further determined the rescue of the effect SOD mimetic, Mn(III)TMPyP (6mg/kg/day, Cayman Chemical, MI) on MCT-induced PAH in SOD3^{E124D} rats. Mn(III)TMPyP treatment started immediately after injections of MCT.

Measurements of aortic pressure and RV hemodynamics

At the end of the study protocol, rats and mice were first anesthetized with 1.5% isoflurane Rats were intubated with a 16-gauge Teflon tube attached to a mechanical ventilator (Kent Scientific-Ventilator, ventilator settings: breathing frequency, 80 breaths per minute; pressures, 9/0 cm H_2O; inspiratory/expiratory ratio, 1:1). Mice were intubated with a 20-gauge Teflon tube attached to MiniVent type 845 mouse ventilator (Hugo Sachs Elektronik).

A 1.2-F pressure catheter (Scisense Inc. Ontario Canada) was introduced through the right common carotid artery into the ascending aorta for measurement of systolic and diastolic blood pressures as described previously.[14,15,17,18] For RV hemodynamics, open-chest RV catheterization was performed during anesthesia with 1.5% isoflurane.[19] Data were collected when steady state was reached.

Sample Preparation

After the final hemodynamic assessment, the rats and the mice were euthanized by exsanguination, and the heart, lung, and other major organs were harvested. Lung weight was determined and the left lung was harvested and snap-frozen in liquid nitrogen for biochemical analysis. The airways of the top right lobe were subsequently perfused with and then fixed in 10% buffered formalin for histological analysis. The wet weight of RV and of left ventricle (LV) + septum (S) were weighed and the ratio of RV weight to LV + S were calculated as an index of RV hypertrophy.[20]

Histological staining, Semiquantification of Fibrosis, and Western Blots

For staining of smooth muscle α-actin, tissue sections (5μm) were deparaffinized, rehydrated, antigen recovered in Tris-EDTA buffer (pH=9.0) 30 minutes at 95–100°C, washed in PBS. The sections were incubated with 3% H_2O_2 in PBS for 20 minutes followed by 1% BSA solution for 1 hour. Sections were then incubated with a monoclonal primary antibody (1:400) against smooth muscle α-actin (Sigma-Aldrich) overnight at 4°C, and followed with Alexa Fluor 555 labeled secondary antibody against mouse Ig-G (1:1000) (Invitrogen).[15] The slides were examined using a confocal microscope (Zeiss LSM510). Measurement of ventricular fibrosis and cardiac myocyte size were performed using the method described previously.[17,21] For methods of **Semiquantification of pulmonary vascular muscularization, fibrosis and Western Blots**, please see http://hyper.ahajournals.org.

Chemical Analysis

Oxidative stress marker TBARS (thiobarbituric acid reactive substances) content was determined as described previously.[14] Total SOD activity and SOD2 activity of lung tissues were measured using a SOD activity kit (Cayman Chemical Company) according the manufacturer's instructions. For relative lung SOD3 activity assay, a total of 2 μg primary antibody for SOD3 (Lifespan Biosciences) was added into 500μl tissue extract (2mg/ml) and then incubated for 1–2 hours at 4° C. Protein A/G-Agarose of 20 μl was then added to the mixture and incubated at 4° C on a rocker platform overnight. The immunoprecipitates were collected by centrifugation at 3,000 rpm for 30 seconds at 4° C. After gently washing with PBS 4 times, the pellets were resuspended in 400μl buffer for SOD activity assay with the commercial kit according to the manufacturer's instructions. Control IgG was used as a negative control.

Total antioxidant capacity

Total antioxidant capacity of lung tissues was determined using an antioxidant power assay kit (Oxford Biomedical Research) according the manufacturer's instructions.

Statistical Analysis

All values are expressed as mean ± standard error or median (± standard error). Data of two groups was compared with unpaired t-test. Two-way analysis of variance was used to test for differences between transgenic and wild type animals under control conditions and after MCT injection. If analysis of variance demonstrated a significant effect, *post hoc* pairwise comparisons were made using the Fisher least significant difference test. Statistical significance was defined as $p<0.05$.

Results

SOD3 knockout aggravated the hypoxia-induced increase of RV pressure and hypertrophy

To study whether SOD3 dysfunction can affect PAH in other experimental models, we determined the effect of SOD3 knockout on hypoxia-induced PAH in mice. RV pressure and the ratio of RV to LV + septum weight were not different between SOD3 knockout mice and wild type controls under control normoxic conditions. However, SOD3 knockout significantly exacerbated hypoxia-induced increases of RV pressure (Figure 1A) and RV hypertrophy as indicated by the ratio of RV to LV+S weight (Figure 1B). In addition, hypoxia caused increases of fully muscularized arterioles in both wild type controls and SOD3 knockout mice, while these increases were significantly greater in the SOD3 knockout mice than in the wild type controls (Figure 1C, Figure S1).

The SOD3^{E124D} mutation had no significant effect on the animals' growth but exacerbated the MCT-induced increase of RV pressure

SOD3^{E124D} rats grew and developed normally. There were no significant differences in terms of body weight gain (Figure 2A), and left ventricular weight between SOD3^{E124D} rats and wild type controls at age of 2 months (Table S1). In addition, systemic blood pressure, heart rate, RV pressure, and the ratio of RV to LV + septum weight were not different between SOD3^{E124D} rats and wild type controls at age of 2 months (Table S1, Table S2, Figure 2B, 2C, 2D, Figure S2, S3). SOD3^{E124D} had no detectable impact on lung SOD3 expression (Figure S4), but significantly reduced lung SOD3 activity ~60% in rats (0.31±0.02 in wild type rats versus 0.12±0.06 in SOD3^{E124D} rats, $p<0.05$).

MCT injection caused significant increase of RV pressure in both SOD3^{E124D} rats and wild type rats (48.6±1.8 mmHg in wild type vs. 57.5±3.1 mmHg in SOD3^{E124D} rats), while MCT caused a significantly greater increase of RV systolic pressure in SOD3^{E124D} rats (Table S2, Figure 2B, 2D), indicating exacerbated pulmonary artery hypertension in SOD3^{E124D} rats. MCT injection significantly attenuated the weight gain in both SOD3^{E124D} and wild type rats (Figure 2A).

The SOD3^{E124D} mutation aggravated the MCT-induced increase of RV hypertrophy and fibrosis

In sham (no monocrotaline) rats at age of 2 months, there was no significant difference between WT and SOD3^{E124D} rats in left ventricle (LV) + septum (S) weight (641±19.3 mg in wild type sham vs. 640±9.4 mg in SOD3^{E124D} rats), right ventricle (RV) weight (162±6.7 mg in wild type sham vs. 157±2.8 mg in SOD3^{E124D} rats), their ratio to body weight or tibia length, and ratio of RV weight to LV + septum weight (0.25±0.02 in wild type untreated sham rats vs. 0.25±0.01 in SOD3^{E124D} untreated sham rats) (Table S1, Figure 2C). Consistent with the significantly greater increase of RV systolic pressure in SOD3^{E124D} rats after MCT, SOD3^{E124D} rats had significantly greater increases of RV weight (239±8.7 mg in wild type rats vs. 285±10.6 mg in SOD3^{E124D} rats, $p<0.05$) and the ratio of RV to LV + septum (0.41±0.01 in wild type rats vs. 0.50±0.09 in SOD3^{E124D} rats, $p<0.05$) in response to MCT (Table S1, Figure 2C), indicating that SOD3^{E124D} mutation exacerbated MCT-induced RV hypertrophy. Histological analysis indicated that MCT caused a significantly greater increase of RV fibrosis (Figure S2, Figure S3A) and cardiac myocyte cross sectional area (Figure S2, Figure S3B), indicating severe RV remodeling in SOD3^{E124D} rats after MCT.

The SOD3^{E124D} mutation exacerbated the MCT-induced pulmonary vascular remodeling

To determine the effect of SOD3^{E124D} on pulmonary vascular remodeling, we determined the percentage of non-muscularized (NM), partially muscularized (PM), and fully

muscularized small arterioles (FM) in wild type rats and SOD3^{E124D} rats under sham conditions and 3 weeks after MCT injection (Figure 3A,B). MCT caused increases of fully muscularized small arterioles both in wild type rats and SOD3^{E124D} rats (55.2 ± 2.3 % in wild type rats vs. 69.9 ± 2.6 % in SOD3^{E124D} rats, $p<0.05$), but these increases were significantly greater in the SOD3^{E124D} rats than in the wild type rats (Figure 3). Meanwhile, MCT also caused decreases of non-muscularized small arterioles both in wild type rats and SOD3^{E124D} rats (9.4 ± 1.12 % in wild type rats vs. 5.2 ± 1.2 % in SOD3^{E124D} rats, $p<0.05$), but these decreases were significantly greater in the SOD3^{E124D} rats than in the wild type rats (Figure 3). In addition, SOD3^{E124D} rats had significantly exacerbated MCT-induced medial wall thickness (Figure 3C) and medial area (Figure S5) of arteries of 50–200um. Together, these data indicate that SOD3^{E124D} significantly exacerbated MCT-induced pulmonary vascular remodeling in rats.

The SOD3^{E124D} mutation exacerbated the MCT-induced pulmonary oxidative stress

MCT also caused increases of pulmonary 3'-nitrotyrosine, and TBARS both in wild type rats and SOD3^{E124D} rats, but these increases were significantly greater in the SOD3^{E124D} rats than in the wild type rats (Figure 4A, 4B, Figure S7), indicating a greater degree of pulmonary oxidative stress in SOD3^{E124D} rats than in wild type rats after MCT. Expression of lung SOD1 and SOD2 were determined (Figure 4C, 4D). SOD3^{E124D} had no detectable impact on lung overall antioxidant capacity as indicated by the power of antioxidants (Figure 4E). Lung total SOD activity was significantly lower in SOD3^{E124D} rats only under control conditions and the difference was small (Figure 4F). Lung SOD2 activity was unchanged in SOD3^{E124D} rats (Figure S8).

SOD mimetic Mn(III)TMPyP rescued SOD3^{E124D} rats from MCT-induced pulmonary artery hypertension: To further determine the impact of lung oxidative stress on the development of PAH, we determined the effect of Mn(III)TMPyP treatment (6mg/kg/day). Mn(III)TMPyP significantly reduced MCT-induced RV pressure (Figure 5A), the ratio of RV weight to LV + septum weight (Figure 5B), and lung vascular remodeling in SOD3^{E124D} rats (Figure 5C,D).

Discussion

SOD3 binds to the surface of endothelial cells and the extracellular matrix and plays a critical role in removing extracellular free radical species. SOD3 is highly expressed in the lung. However, as SOD3 has a minimal impact on total tissue SOD activity,[10] it is uncertain whether endogenous SOD3 can influence the development of PAH. In the present study, we report that SOD3^{E124D} had no effect on overall pulmonary oxidative stress, PAH and RV hypertrophy under control conditions but resulted in more severe pulmonary hypertension, more remodeling of the pulmonary arteries and more right ventricular hypertrophy and fibrosis in the setting of MCT-induced pulmonary hypertension. In addition, SOD3 knockout aggravated hypoxia-induced PAH in mice. The findings indicate that endogenous SOD3 plays an important role in protecting the lung from the development of PAH under stress conditions.

The over-expression of extracellular SOD3 reduces both hypoxia- and monocrotaline-induced PAH.[11,12] Similarly, an increase in SOD3 activity decreases hypoxic pulmonary vasoconstriction in bovine pulmonary artery rings.[22] Loss of SOD3 could result in increased levels of O^{2-} and down-stream radicals such as peroxynitrite, decreased H_2O_2 in extracellular space, and reduced intercellular diffusion of endothelial NO to surrounding cell types. Decreased NO bioavailability enhances the development of PAH, while increased NO or increase of its down-stream product cGMP by inhibition of PDE5 attenuate the development of PAH.[23] Thus it is possible that a contributing factor to the exacerbated PAH

in $SOD3^{E124D}$ rats and SOD3 knockout mice is increased scavenging of NO by superoxide and a subsequent reduction in NO/cGMP bioavailability.

The role of endogenous SOD3 in pulmonary vascular physiology and pathophysiology has not been clear. In these studies we show that loss of SOD3 function does not affect the tone or structure of the pulmonary arteries under normoxic control conditions. The finding that loss of function mutation in $SOD3^{E124D}$ rats and SOD3 knockout in mice results in more severe pulmonary hypertension and more RV hypertrophy after MCT or hypoxia (but not under control conditions) is conceptually consistent with previous studies that loss of SOD3 function exacerbated infarction or pressure overload-induced left ventricular maladaptive remodeling.[14,24] Thus, O^{2-} and possibly related down-stream radicals, play a detrimental role in the pathophysiology of PAH as well as other pathological conditions such as ventricular remodeling.[25,26]

The overall role of SOD and oxidative stress are of particular interest in the pulmonary vasculature because the expression of SOD2 is found to be reduced in IPAH patients and in fawn-hooded rats that spontaneously develop PAH.[27] The decrease in SOD2 precedes the development of PAH in the fawn-hooded rats. The use of an SOD mimetic prevents pulmonary hypertension and reduces right ventricular hypertrophy in rats exposed to chronic hypoxia[28] and in fawn-hooded rats.[4] Similar to the effects of SOD3 depletion, a reduction in SOD2, should also increase O^{2-} levels and down-stream radicals such as peroxynitrite, and decrease levels of H_2O_2. SOD2 and SOD3 thus appear to play a major role in protecting against the development of PAH through decreasing O^{2-} and increasing H_2O_2 and NO bioavailability.[29] These results suggest that SOD mimetics or treatments that increase endogenous SOD2 or SOD3 may have therapeutic value in PAH.

Clinical Perspective

Pulmonary artery hypertension (PAH) is a progressive disease with a very poor prognosis. PAH is characterized by a progressive elevation of pulmonary arterial pressure, ultimately inducing right ventricular (RV) hypertrophy and heart failure. Studies have demonstrated that increased oxidative stress may contribute to the pathogenesis and the development of idiopathic PAH. Extracellular superoxide dismutase (SOD3) plays an important role in attenuating superoxide anion in the extracellular space. However, the effect of the endogenous SOD3 on the development of PAH has not been clear. Here we report that both SOD3 knockout in mice or $SOD3^{E124D}$ mutation in rats resulted in significantly greater increases of RV pressure, RV hypertrophy and pulmonary vascular remodeling in response to hypoxia (in mice) or monocrotaline (in rats). The findings indicate that endogenous SOD3 plays an important role in protecting against the development of PAH and subsequent RV hypertrophy under stress conditions. These results suggest that SOD mimetics or treatments that increase endogenous SOD3 may have therapeutic value in PAH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding: This study was supported by NHLBI Grants R21HL102597 (YC), R21HL098719 (YC), R21HL098669 (YC), HL079168 (RJB) and HL65322 (EKW) from the National Institutes of Health, GRNT2260175 (YC) and a Scientist Development Award from American Heart Association (XH), and VA Research funding (EKW).

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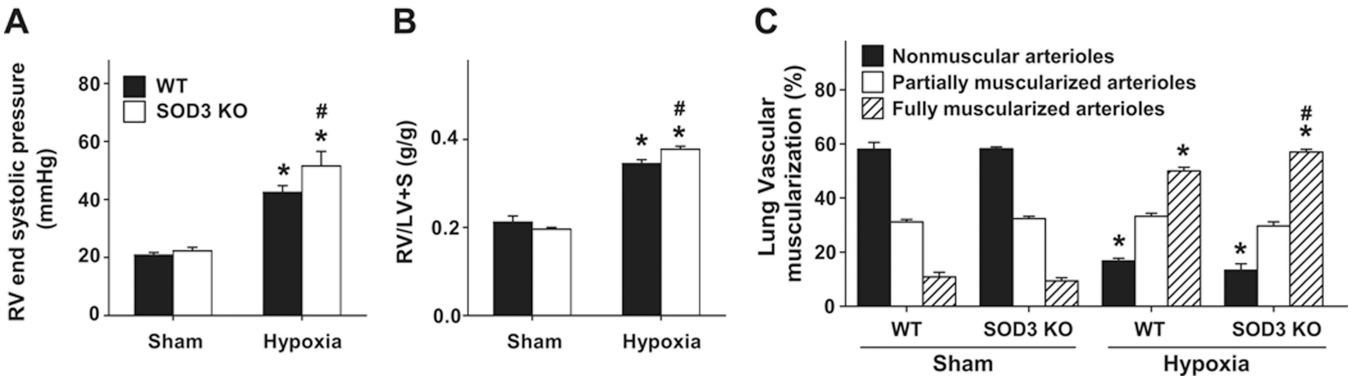

Figure 1.

SOD3 knockout in mice significantly exacerbated hypoxia-induced increases of RV pressure (A), of RV hypertrophy as indicated by the ratio of RV to LV+S weight (B), and of pulmonary vascular remodeling as indicated by significant increases of fully muscularized small arterioles (C). *p<0.05 vs sham control; #p<0.05 vs corresponding WT mice.

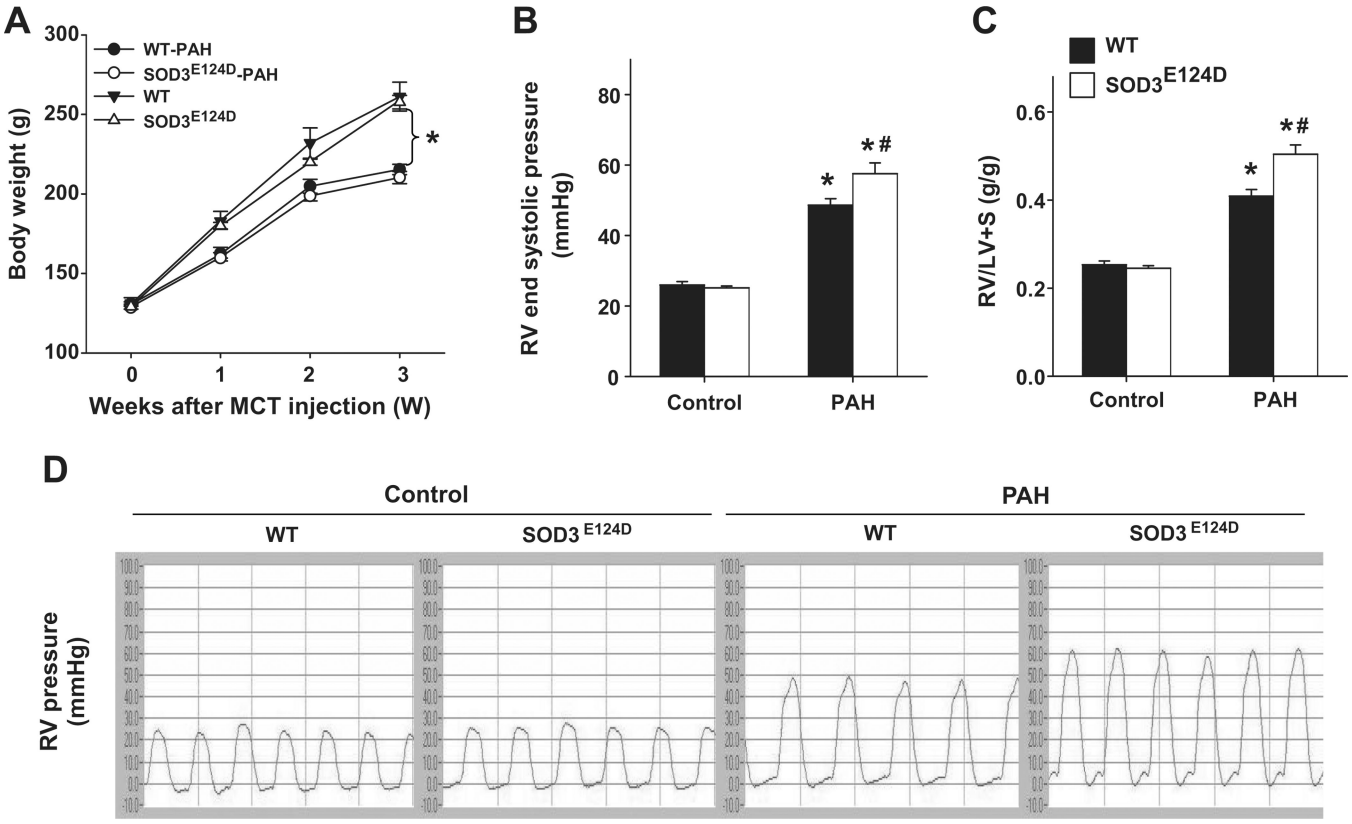

Figure 2.

$SOD3^{E124D}$ mutation in rats had no significant effect on growth but exacerbated MCT-induced increase of RV pressure. $SOD3^{E124D}$ mutation had no effect on growth under both control conditions and after MCT injection respectively (A). $SOD3^{E124D}$ mutation had no effect on RV pressure under control conditions but further elevated the MCT-induced increase of RV pressure, indicating more severe PAH in $SOD3^{E124D}$ rats (B,C). *p<0.05 vs sham control; #p<0.05 vs. corresponding WT rats.

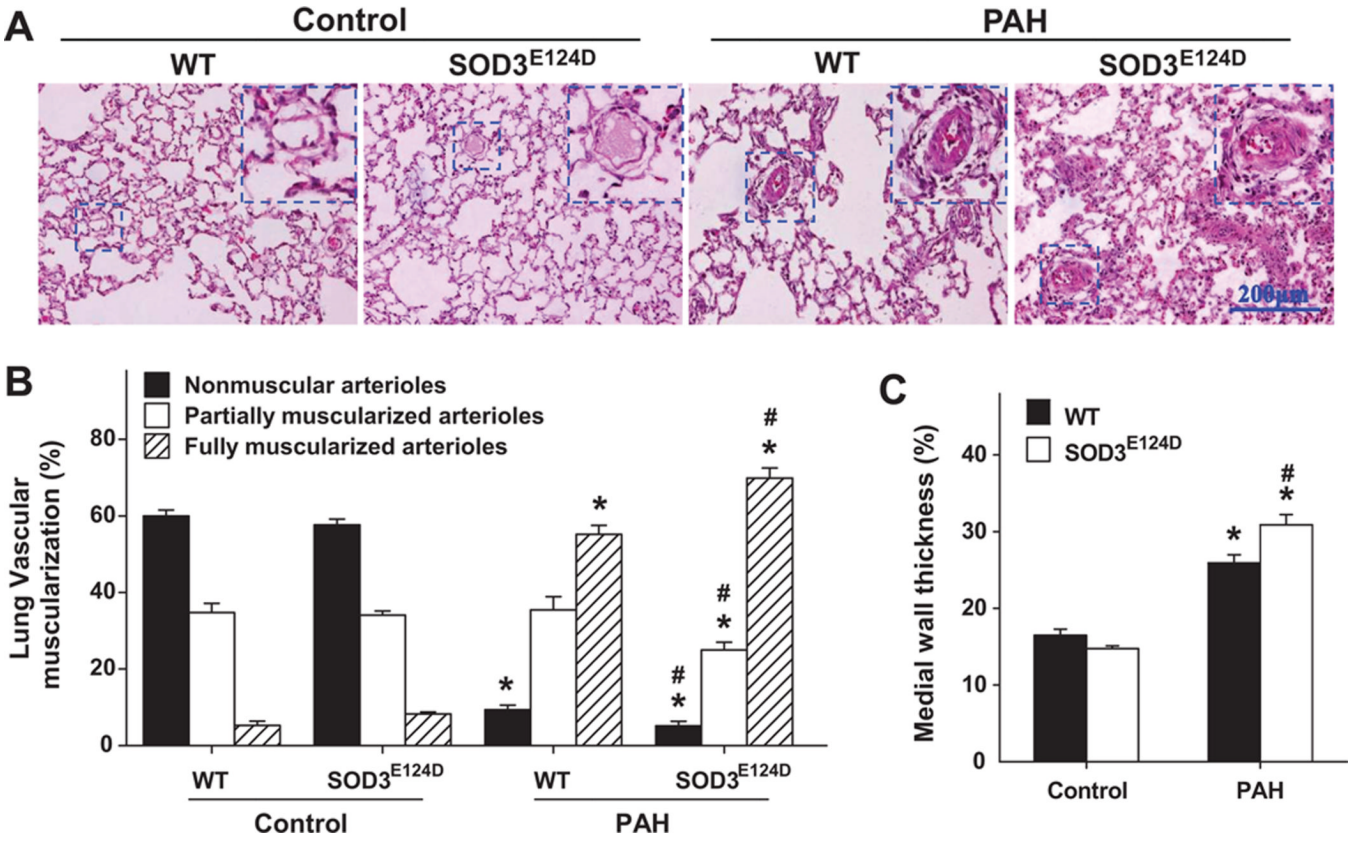

Figure 3.

SOD3^{E124D} mutation caused significantly greater pulmonary vascular remodeling. Distribution of nonmuscular, partially muscular, and fully muscularized small arterioles in WT rats and MCT-induced PAH rats (A,B). SOD3^{E124D} significantly aggravated MCT-induced pulmonary vascular muscularization (B). SOD3^{E124D} mutation significantly exacerbated MCT-induced relative medial wall thickness of larger arteries in rats (C). *p<0.05 vs sham control; #p<0.05 vs corresponding WT rats.

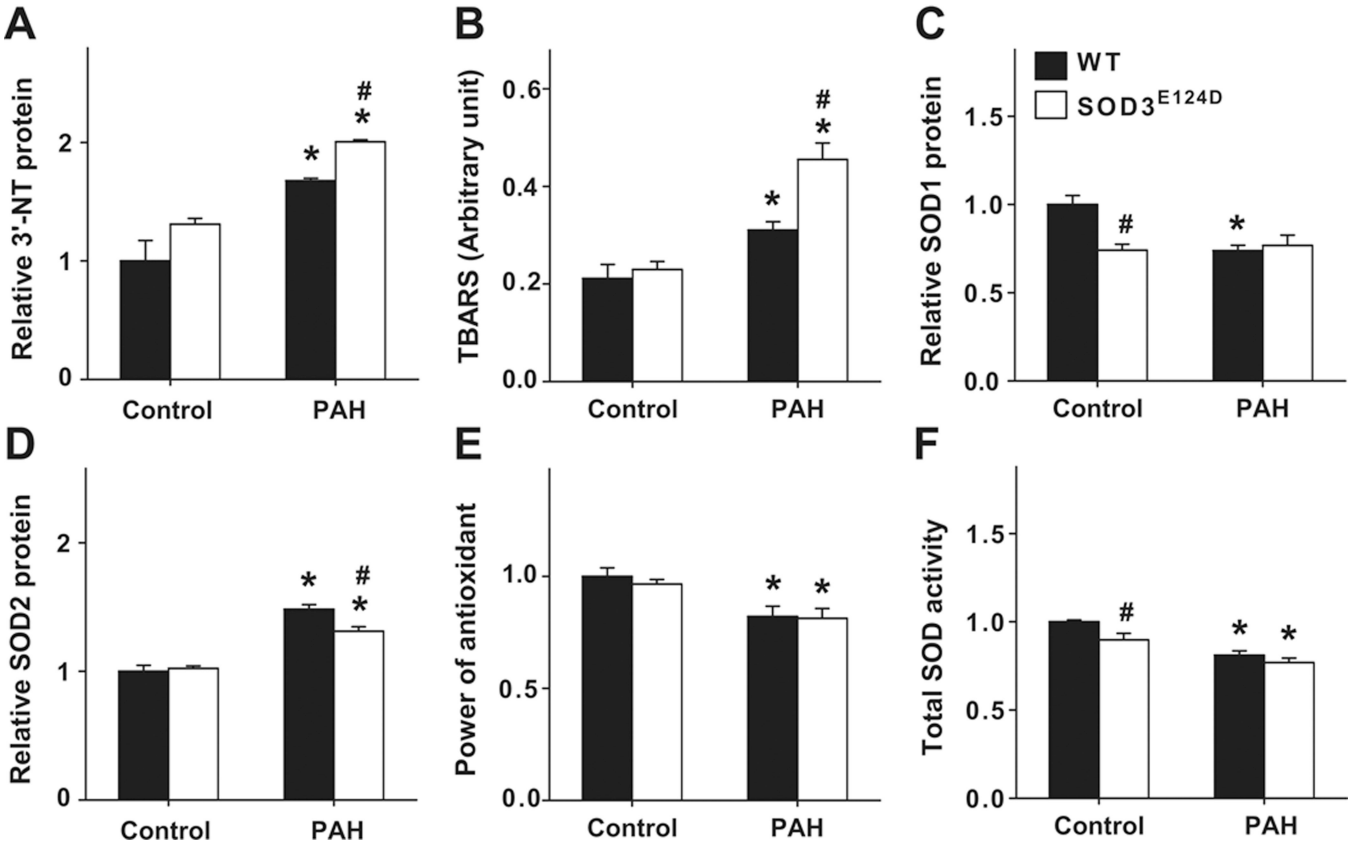

Figure 4.

SOD3^{E124D} mutation caused significantly greater increase of lung oxidative stress. Increases of oxidative stress markers 3'-nitrotyrosine and TBARS (A,B) were observed in SOD3^{E124D} rats. Changes were also noted in the expression of SOD1 protein (C), SOD2 protein (D), and total SOD activity (E) in SOD3^{E124D} and wild type rats under control conditions or after MCT injection. No change was seen in the overall antioxidant capacity (F) *p<0.05 vs sham control; #p<0.05 vs corresponding WT rats.

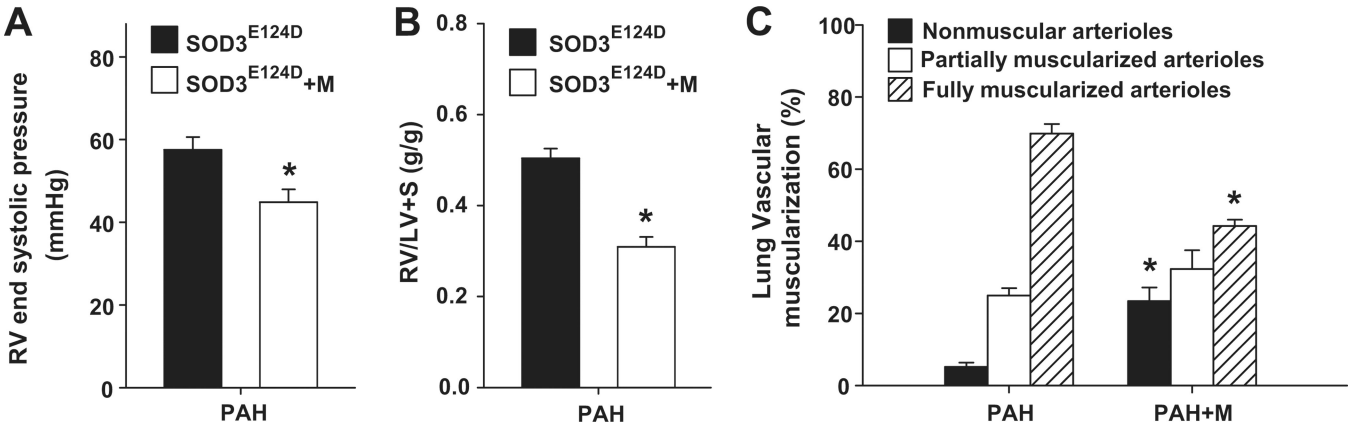

Figure 5.

SOD mimetic Mn(III)TMPyP significantly rescued SOD3^{E124D} rats from MCT-induced increase of RV pressure (A), RV hypertrophy (B) and pulmonary vessel remodeling (C). *p<0.05 vs rats with MCT injection alone.